Supporting Information

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SI Materials and Methods

Immune-Precipitation and Antibodies Used in this Study

Antibodies. The primary antibodies used were as follow: anti-S473P-Akt, anti-T308P-Akt, anti-pHER2, anti-pHER3, anti-HER2, pERK1/2, anti-S235/6-RPS6, anti-S240/244-RPS6, anti-PTEN, anti-total and cleaved PARP, anti total and cleaved caspase-3, anti-cleaved capase-6, anti-cleaved capase-7, anti-total and cleaved capase-9, anti-total and cleaved caspase-8, anti-total caspase-10 antibodies were from Cell Signaling Technologies; the anti-Akt and anti-total and cleaved caspase-2 were from Epitomics and Abcam, respectively.

p85 Immunoprecipitation. Fifteen hundred micrograms total cell lysates was immunoprecipitated overnight in a total volume of 750 μL of lysis buffer, with anti-p85 antibody (Millipore, diluted 1:50), and a mix of 50 μL protein G-Sepharose plus 50 μL protein A-Sepharose (GE Healthcare). Next day, the immune-precipitates were washed twice the with lysis buffer and analyzed by Western blotting.

In Vivo Studies with DC101. The Rat IgG1 anti-vascular endothelial growth factor receptor-2 antibody DC101 (ImClone Systems, Inc) was prepared in PBS as a 9.9 mg/mL stock solution. For efficacy studies, DC101 was given i.p., every 3 days, at a dose of 42 mg/kg, in PBS, with an application volume of 10 mL/kg.

Preparation of Tumor Section for Immunohistochemistry. Tumors were fixed immediately after dissection in 10% neutral buffered formalin solution for exactly 24 h at 4 °C. After fixation, dissected tumors were rinsed in PBS and processed for dehydration, clearing, and paraffinization under vaccuum conditions in the TPCduo apparatus (Medite) as described above. The tumors were then embedded in paraffin, and 3-μm sections prepared, mounted on polysine coated microscope slides, and dried at 37 °C for 16 h.

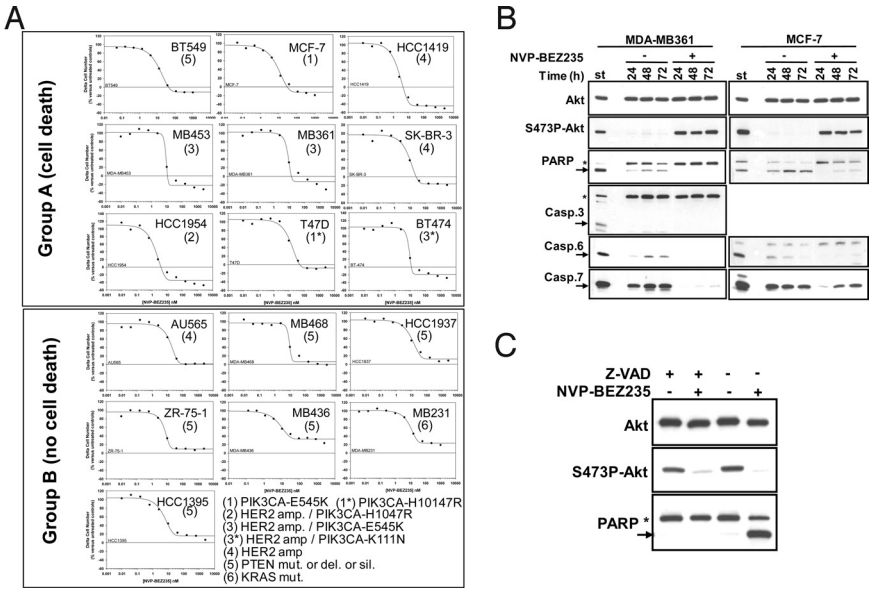

Fig. S1. Antiproliferative and cell death induction activities of NVP-BEZ235 in breast cancer cell lines comparison with pure PI3K, Akt, and mTORC1 inhibitors. The mentioned breast cancer cell lines were incubated with increasing amount of NVP-BEZ235 for a period of 72 h. Cells were then fixed and effect on viability recorded and plotted as a difference versus the growth measured in control untreated cells (100%). The 0% percentage represents complete suppression of proliferation, and negative values, reflects reduction of cell number hence active cell killing. Based on the obtained fit curves position with regards to the 0% line, cell lines were clustered either in Group A (sensitive, cell death observed) or Group B (insensitive, no cell death observed). (*B*) MDA-MB361 or MCF-7 cells were incubated either with 1 μM of NVP-BEZ235 for the indicated time or for 3 h with 1 μM of staurosporine (st), and processed as in Fig. 1B for the detection of the indicated markers. (*C*) MCF-7 cells were incubated for 24 h with both 1 μM of NVP-BEZ235 and 200 μM ZVAD. Legend: *, uncleaved form; arrow, cleaved form; [(1)], PIK3CA mut.; [(2)], HER2 amp./IK3CA-H1047R; [(3)], HER2 amp./PIK3CA-E545K; [(4)], HER2 amp; [(5)], PTEN alterations; [(6)], KRAS mut. This legend applies to all of the figures in *SI Materials and Methods*.

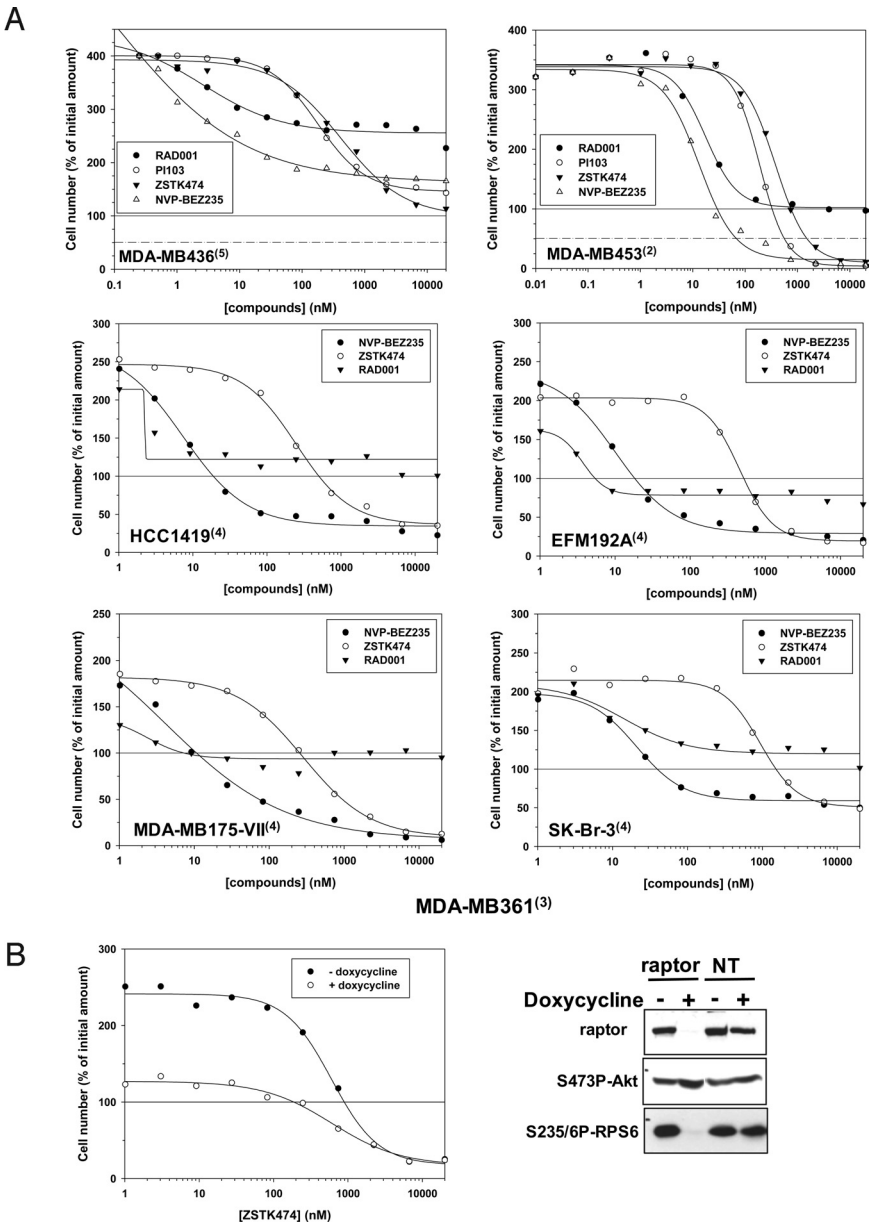

Fig. S2. Anti-proliferative and cell death induction activities of pure PI3K, Akt and mTORC1 inhibitors in breast cancer cell lines. (A) The tumor cell lines MDA-MB453, MDA-MB436, Sk-Br-3, HCC1419, EFM192A, and MDA-MB175-VII were incubated with increasing concentrations of the indicated compounds for a period of 72 h. Cells were then fixed and effect on viability recorded and plotted as a percentage of the number of cells present at the time from which the cells were exposed to the compound (100% straight line). The dashed line represent the concentration for which the compound is able to kill 50% of the cells (dashed line − LD_{50} values). (*B*) Raptor protein knock-down was induced (raptor) or not (NT for non targeting shRNA) in the HCC1954 cell line by the addition of doxycycline (200 ng/mL) to the growth media. Seventy-two hours later, the cells were analyzed for the effects on proliferation (*Left*) and signaling events (*Right*), either in absence or presence of the indicated amounts of the PI3K inhibitor ZSTK474.

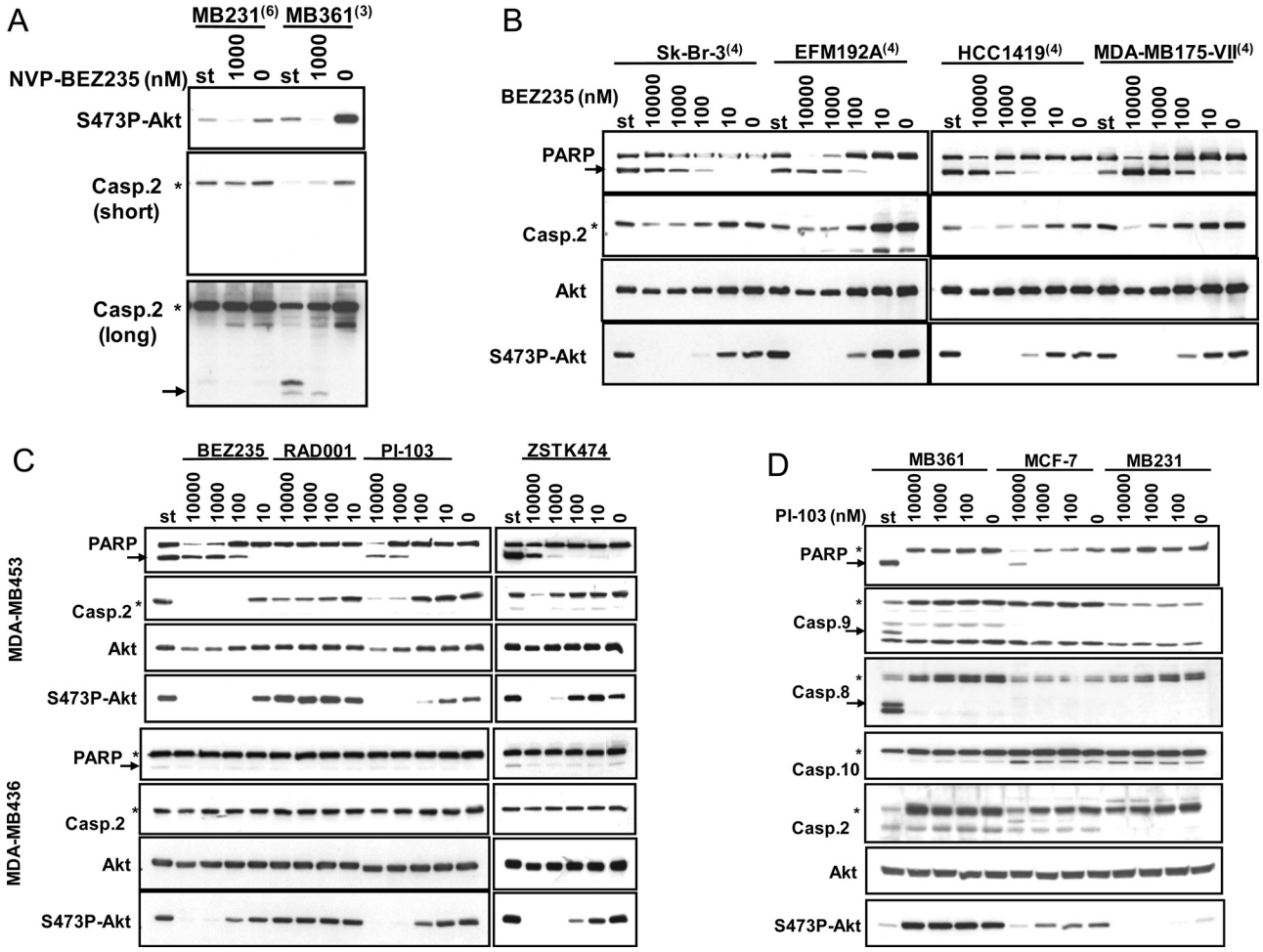

Fig. S3. NVP-BEZ235 and the pure PI3K inhibitors but not RAD001 induces caspase-2 cleavage. (*A*) MDA-MB231 and MDA-MB361 cells were incubated either for 48 h with 1 μM of NVP-BEZ235 or for 3 h with 1 μM of staurosporine (st). Cells were then lysed and cell extracts analyzed by Western blotting for the detection of S473P-Akt levels, or total (*, short exposure) and cleaved forms of caspase-2 (arrow, long exposure). (*B–D*) Sk-Br-3 (*B*), EFM192A (*B*), HCC1419 (*B*), MDA-MB175-VII (*B*), MDA-MB453 (*C*), MDA-MB436 (*C*), MDA-MB361 (*D*), MCF7 (*D*), and MDA-MB231 (*D*) cells were incubated for 48 h with the indicated amount (in nM) of either NVP-BEZ235 (*B* and *C*), RAD001 (*C*), PI-103 (*C* and *D*), and ZSTK474 (*C*) or for 3 h with 1 μM of staurosporine (st). Cells were then lysed and cell extracts analyzed by Western blotting for the detection of the indicated markers.

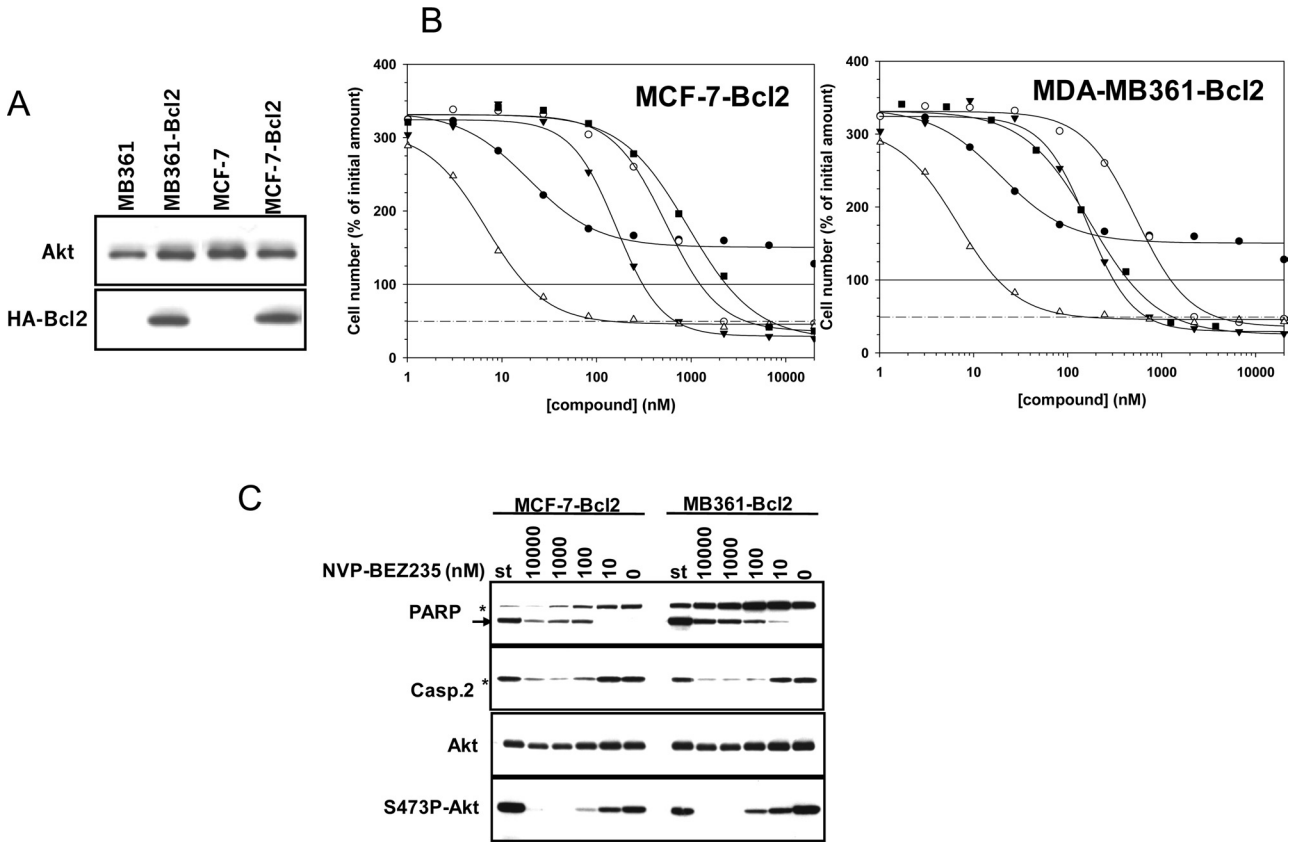

Fig. S4. Apoptosis induction by the PI3K inhibitors involves the executioner caspase. caspase-2. (*A*) MDA-MB361 and MCF-7 cells were transfected with the pcDNA3.1-HA-Bcl2 vector, and pools stably expressing the HA-tagged Bcl2 protein selected. (*B*) MDA-MB361 and MCF7-Bcl2 over-expressing cell lines were incubated with increasing concentrations of either, RAD001, PI-103, ZSTK474, NVP-BEZ235, or C124017 for a period of 72 h. Cells were then fixed and effect on viability plotted as described in Fig. S1*A*. The dashed line represents the concentration for which the compound is able to kill 50% of the cells (LD_{50} values). (*C*) MDA-MB361 and MCF7-Bcl2 over-expressing cell lines were incubated either for 48 h with the indicated amount (in nM) of NVP-BEZ235 or for 3 h with 1 μM of staurosporine (st). Cell extracts were then analyzed for the indicated markers.

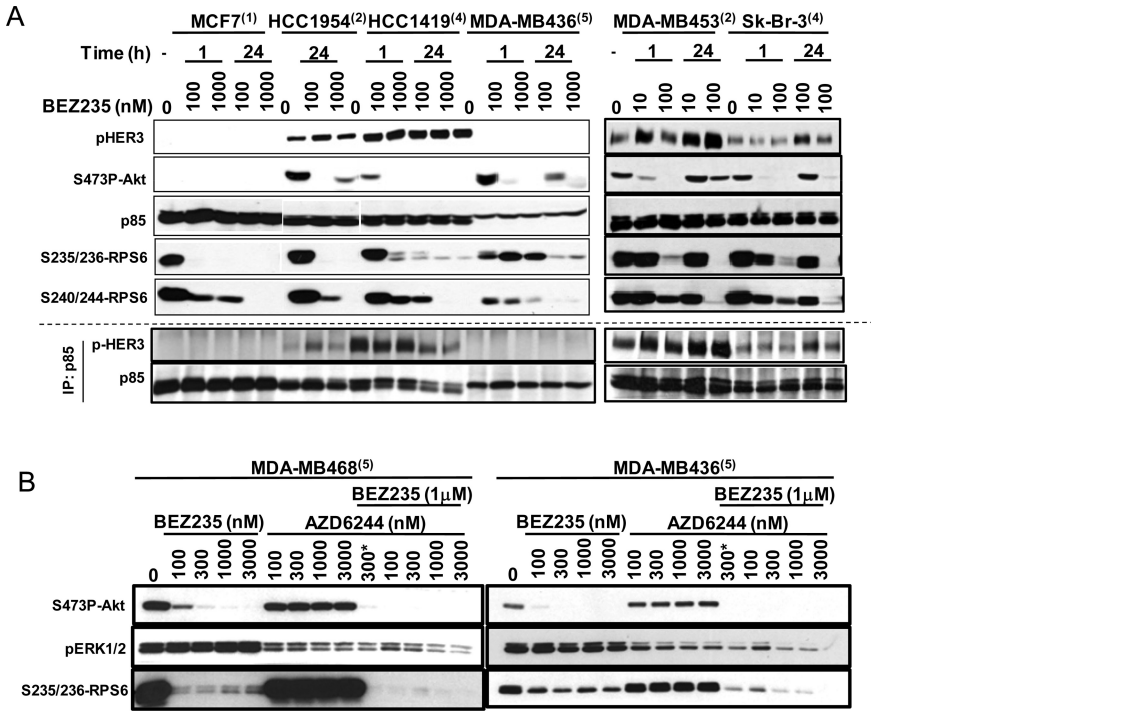

Fig. S5. NVP-BEZ235 activities in HER2 amplified and PTEN deficient breast cancer lines. (*A*) The indicated cell lines were pretreated for 1 or 24 h with the indicated amount of BEZ235 and the corresponding extracts analyzed either directly (lysates) or indirectly after immune-precipitation with anti-p85 immobilized beads (IP: p85, as described in *Materials and Methods*) for their pHER3, activated Akt, and activated RPS6 levels. (*B*) the PTEN negative lines MDA-MB468 (*Left*) and MDA-MB436 (*Right*) were incubated with either BEZ235 alone, or with the MEK inhibitor AZD6244 alone or with both molecules, at the indicated concentrations for 1 h. The corresponding cell extracts were then analyzed for their activated Akt, ERK1/2, and RPS6 levels.

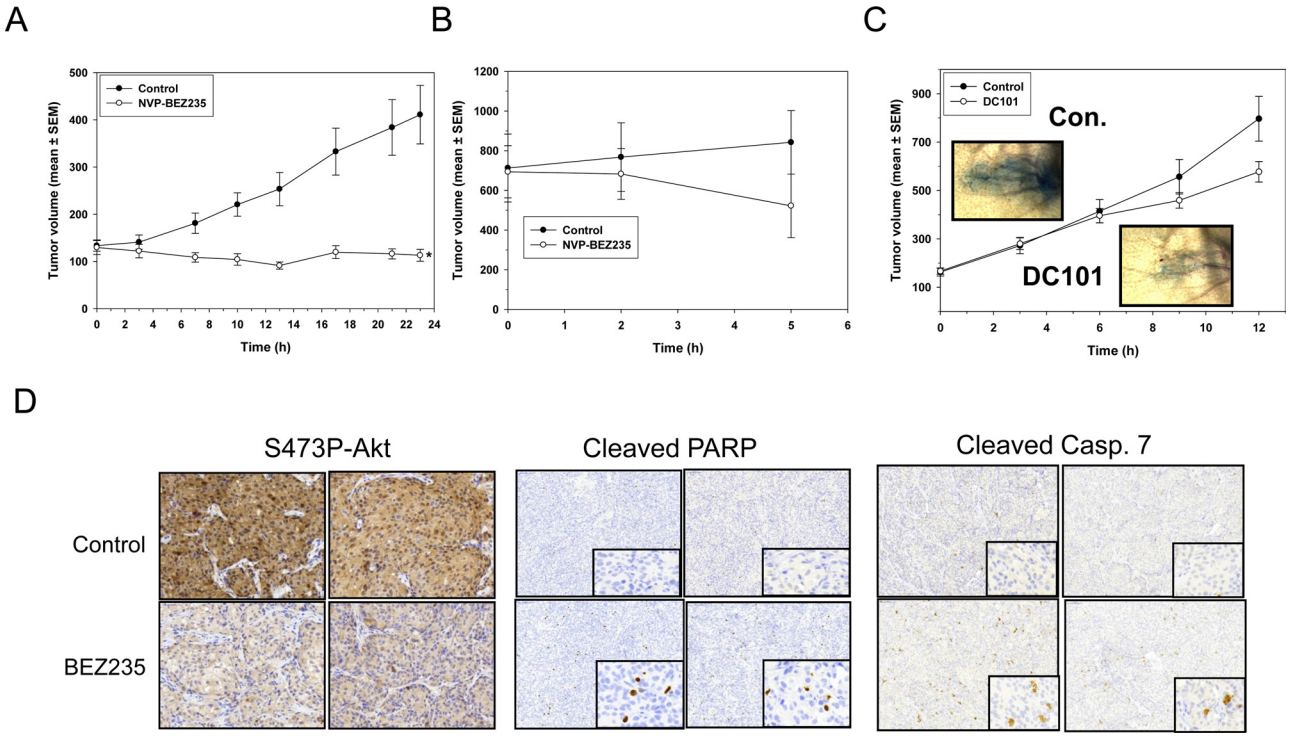

Fig. S6. NVP-BEZ235 antitumor activity is not primarily due to pure anti-angiogenic effects. (*A* and *B*) MCF7 (*A*) and MDA-MB453 (*B*) orthotopic tumor bearing animals were treated p.o., once per day, either with 45 mg/kg of NVP-BEZ235 or with the vehicle control. Tumor volumes were recorded during the treatment period and T/C values were calculated (see *Results*). (*C*) Mice bearing orthotopic MDA-MB361 tumors were treated i.p., every 72 h, either with 42 mg/kg of DC-101 or with the vehicle control and tumor volumes were recorded during the treatment period. One hour after the last dose, three animals from both groups were subjected to a modified Miles assay. The extravasation of the Evan's blue dye in the ear of the animals was then photographed (*Inset*). (*D*) At culling time, the tumors from the efficacy study described in Fig. 5*A* (MDA-MB361 tumors) were dissected, cut in two equal pieces. Half of the tumors were snapped freeze in liquid nitrogen and processed for Western blot analysis (see Fig. 5*B*). The remaining half was processed for detection by IHC of S473P-Akt, cleaved PARP and cleaved caspase-7.

Table S1. Activities of NVP-BEZ235 in breast cancer cells

Cell line	Genotype				GI_{50} (nM)	Group	LD_{50} (nM)
	PIK3CA	PTEN	K-Ras	EGFR			
MCF7	E545K		—	—	6.6	A	117/69/76
HCC1419			—	ErbB2amp	7.3	A	55/101
HCC1954	H1047R		—	ErbB2amp	8.5	A	126
MDA-MB361	E545K		—	ErbB2amp	6.0	A	31/37/28
MDA-MB453	H1047R		—	ErbB2amp	8.3	A	53/68/44
BT474	K111N		—	ErbB2amp	7.5	A	nd
Sk-Br-3			—	ErbB2amp	8.6	A	>20,000*
BT549		del	—	—	9.9	A	nd
T47D	H1047R		—	—	9.6	A	nd
EFM192A				ErbB2amp	6	A	81/71
MDA-MB-175-VII				ErbB2amp	3	A	75/73
AU565			—	ErbB2amp	12.6	B	nd
MDA-MB231			mut	—	9.2	B	>20,000
MDA-MB468		del	—	—	12.6	B	nd
HCC1395		del	—	—	17.1	B	nd
ZR-75–1		mut	—	—	17.4	B	nd
HCC1937		del	—	—	21.2	B	nd
MDA-MB436		sil	—	—	26.5	B	>20,000

The mentioned cell lines and their described genetic status for the indicated genes were gown in vitro and used to determine the antiproliferative (GI_{50}) and cell death induction (LD_{50}) properties of NVP-BEZ235, as described in the *Material and Method* section. del, deletion; amp, amplification; sil, silenced; mut, mutated, nd, not determined.

*, cell death observed but LD50 not achieved in the range of concentration tested.

Table S2. Reported and determined IC50 values for class IA PI3Ks, Akt, PDK1 and mTOR enzymes

	NVP-BEZ235	ZSTK474	PI-103	C124017
p110α	4*	16[†]	2[‡]	>10000[§]
p110β	75*	44[†]	3[‡]	>10000[§]
p110δ	7*	4.7[†]	3[‡]	>10000[§]
Akt1	>10,000	>10,000	>10,000[‡]	58[§]
PDK1	>10,000	>10,000	>10,000[‡]	210[§]
mTOR	4 ± 2	>9,100	252/270	nt
S2356/236-RPS6	6.5*	1,690	436	nt

The activities against PI3K, Akt1, and PDK1 were as described in the references listed below. NVP-BEZ235, ZST474, PI-103 and C124017 were tested also for their ability to inhibit mTOR in vitro, or S235/236-RPS6 in TSC1 null MEFS, as described in ref. 1*.

*1. Maira SM, et al. (2008) Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity. *Mol Cancer Ther* 7:1851–1863.

[†]2. Kong D, Yamori T (2007) ZSTK474 is an ATP-competitive inhibitor of class I phosphatidylinositol 3 kinase isoforms. *Cancer Sci* 98:1638–1642.

[‡]3. Raynaud FI, et al. (2007) Pharmacologic characterization of a potent inhibitor of class I phosphatidylinositide 3-kinases. *Cancer Res* 67:5840–5850.

[§]4. Bilodeau MT, et al. (2008) Allosteric inhibitors of Akt1 and Akt2: A naphthyridinone with efficacy in an A2780 tumor xenograft model. *Bioorg Med Chem Lett* 18:3178–3182.